This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

THIS PAGE BLANK (USPTO)

EXHIBIT 10

J.D. FIKES ET AL

J. BIOL. CHEM. 265 3417-3423, 1990

THIS PAGE BLANK (USPTO)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1990 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 265, No. 6, Issue of February 25, pp. 3417-3423, 1990
Printed in U.S.A.

EXHIBIT IC

Maturation of *Escherichia coli* Maltose-binding Protein by Signal Peptidase I *in Vivo*

SEQUENCE REQUIREMENTS FOR EFFICIENT PROCESSING AND DEMONSTRATION OF AN ALTERNATE CLEAVAGE SITE*

(Received for publication, July 27, 1989)

John D. Fikes‡, Genevieve A. Barkocy-Gallagher, David G. Klapper, and Philip J. Bassford, Jr.§

From the Department of Microbiology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7290

Comparative analyses of a number of secretory proteins processed by eukaryotic and prokaryotic signal peptidases have identified a strongly conserved feature regarding the residues positioned −3 and −1 relative to the cleavage site. These 2 residues of the signal peptide are thought to constitute a recognition site for the processing enzyme and are usually amino acids with small, neutral side chains. It was shown previously that the substitution of aspartic acid for alanine at −3 of the *Escherichia coli* maltose-binding protein (MBP) signal peptide blocked maturation by signal peptidase I but had no noticeable effect or MBP translocation across the cytoplasmic membrane of its biological activity. This identified an excellent system in which to undertake a detailed investigation of the structural requirements and limitations for the cleavage site. *In vitro* mutagenesis was used to generate 14 different amino acid substitutions at −3 and 13 different amino acid substitutions at −1 of the MBP signal peptide. The maturation of the mutant precursor species expressed *in vivo* was examined. Overall, the results obtained agreed fairly well with statistically derived models of signal peptidase I specificity, except that cysteine was found to permit efficient processing when present at either −3 and −1, and threonine at −1 resulted in inefficient processing. Interestingly, it was found that substitutions at −1 which blocked processing at the normal cleavage site redirected processing, with varying efficiencies, to an alternate site in the signal peptide represented by the Ala-*X*-Ala sequence at positions −5 to −3. The substitution of aspartic acid for alanine at −5 blocked processing at this alternate site but not the normal site. The amino acids occupying the −5 and −3 positions in many other prokaryotic signal peptides also have the potential for constituting alternate processing sites. This appears to represent another example of redundant information contained within the signal peptide.

Most secretory proteins are initially synthesized with an amino-terminal extension designated the signal peptide which serves to initiate export of the protein and which is subsequently removed during or immediately following translocation (1, 2). Most signal peptides share common structural features: a hydrophilic amino-terminal segment with 1-3 basic residues followed by a 9-15-residue core of hydrophobic and neutral amino acids and finally a more polar carboxyl terminus that immediately precedes the cleavage site (for review, see 3). According to the loop model (4), the positively charged amino terminus interacts with the membrane surface, and the hydrophobic core inserts into and spans the lipid bilayer as a loop or reverse hairpin structure, exposing the cleavage site on the external face. Many details of this model remain a matter of conjecture; still, the experimental evidence supports this orientation of the signal peptide during translocation (1, 5, 6). The enzymes responsible for the endoproteolytic removal of the signal peptide are termed signal peptidases. Eukaryotic signal peptidase has been purified from several tissue sources and is an integral membrane complex of two to six polypeptides (7, 8). Two distinct signal peptidases have been purified from *Escherichia coli* cells (9). Both are integral membrane proteins composed of a single polypeptide. Signal peptidase II cleaves the signal peptides of lipoproteins exclusively, and signal peptidase I processes all other secretory proteins.

Although signal peptides exhibit little primary sequence homology, the comparative analysis of numerous prokaryotic and eukaryotic proteins has identified a strongly conserved feature regarding the residues at positions −3 and −1 relative to the cleavage site. It was noted that amino acids with small, neutral side chains predominated at these two positions (10, 11). von Heijne (12) compared 36 prokaryotic signal peptides processed by signal peptidase I, and only alanine, glycine, leucine, serine, threonine, and valine were encountered at the −3 position. Somewhat more restrictive, position −1 was found to harbor only alanine, glycine, serine, and threonine. Ala-*X*-Ala is the most frequently observed sequence preceding the cleavage site (10, 11). Based on these frequency analyses, positions −3 and −1 have been proposed to constitute a recognition site for the processing enzyme. These observations also were utilized to formulate the A-*X*-B model (10) and the (−3,−1) rule (11) for predicting signal peptide cleavage sites. The (−3,−1) rule was later further modified by incorporating a larger statistical basis and a weighted matrix approach (12). Various studies with *E. coli* have provided support for the basic tenets of these statistically based models (6, 13-15). However, such studies did not include a systematic analysis of multiple substitutions at −3 or −1 to determine experimentally the requirements and limitations for efficient signal peptide processing.

* This research was supported by Grant AI17292 from the National Institute of Allergy and Infectious Diseases. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Biology 56-420, Massachusetts Institute of Technology, Cambridge, MA 02139.

§ To whom correspondence should be addressed. Tel.: 919-966-1034.

The MBP[1] signal peptide is composed of 26 amino acid residues and exhibits all the features typical of prokaryotic signal peptides, including alanine residues at both −3 and −1 (16). The substitution of aspartic acid for alanine at −3 was found to block MBP processing by signal peptidase I without having a noticeable effect on translocation across the cytoplasmic membrane (6). The preMBP remained anchored to the cytoplasmic membrane by its unprocessed signal peptide but was still capable of facilitating maltose uptake efficiently (6, 17). The characterization of this mutant MBP species served to underscore the nonoverlapping nature of the hydrophobic core and processing region and identified an excellent system to conduct mutational analysis of cleavage site structural requirements with affecting protein translocation. In this study, oligonucleotide-directed mutagenesis has been utilized to construct plasmids encoding an assortment of mutant preMBP species altered at specific positions near the signal peptide processing site. The effect of 14 unique single amino acid substitutions at −3 and 13 unique substitutions at −1 on processing were examined and the results compared with predictions based on statistically derived models of signal peptidase I specificity. Interestingly, certain changes at −1 uncovered a nearby alternate cleavage site in the preMBP.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—*E. coli* K-12 strain BAR1091 (F′ *lacI*^{q} Tn*5*/*malE*Δ*312 lacI*^{q} *thi relA araD139 flbB pstF rpsL*) (18) was used for all aspects of this study except preparation of single-stranded templates for mutagenesis (see below). The *malE*Δ*312* mutation is an in-frame, nonpolar deletion that removes DNA sequences encoding residue 15 of the MBP signal peptide through residue 159 of the mature MBP. Plasmid pJF2 is a derivative of pBR322 harboring the *malE* gene encoding MBP under lacUV5 promoter-operator control and the M13 intergenic region (6). The latter permits packaging of single-stranded plasmid DNA upon infection with the helper bacteriophage M13KO7 (19). Plasmid pJF8 is a derivative of pJF2 which harbors an amber mutation at the codon corresponding to position 23 of the MBP signal peptide (6). Plasmid pJF13 also is a derivative of pJF2 encoding MBP with aspartic acid substituted for alanine at residue −3 (6).

Reagents—Minimal medium M63 supplemented with carbon source (0.2%) and thiamine (2 μg/ml), maltose tetrazolium indicator agar, and TYE agar were prepared as described previously (20). When required, ampicillin was added to minimal and complex media at concentrations of 25 and 50 μg/ml, respectively. To induce *malE* genes under *lacUV5* promoter-operator control, IPTG was used on agar plates and in liquid media at 1 and 5 mM, respectively. [^{35}S] Methionine (translation grade; 1154 Ci/mmol) was obtained from Du Pont-New England Nuclear. Rabbit anti-MBP serum has been described previously (21). Electrophoresis reagents, T4 DNA ligase, and large fragment of DNA polymerase I were purchased from Bethesda Research Laboratories. Kodak XAR film was obtained from Eastman Kodak.

Oligonucleotide-directed Mutagenesis of malE—To introduce mutations into *malE*, the gene encoding MBP, the oligonucleotide-directed mutagenesis method of Zoller and Smith (22) was used with the following changes: (i) single-stranded plasmid DNA was used as template; (ii) to increase the efficiency of mutagenesis as described by Kunkel *et al.* (23), uracil-containing templates were prepared from cells of *E. coli* strain CJ236 (*ung*$^-$ *dut*$^-$); and (iii) in order to randomize hybridization of the mixed oligonucleotides, the ratio of template to oligonucleotide was reduced to 1:1 or 2:1, and the annealing reactions were slowly cooled from 70 °C to room temperature. Mutagenic primers were prepared with an Applied Biosystems 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis as described by Hutchison *et al.* (24).

The oligonucleotides used to mutagenize *malE* at the codons corresponding to either −3 (designated PS.2) or −1 (PS.7) of the si peptide are shown in Fig. 1. Each oligonucleotide was complemen to the DNA strand of packaged, single-stranded pJF8, and encoded the wild-type sequence except at the first 2 bases of codon to be mutagenized. The mutagenic oligonucleotides repl the amber mutation (TAG) at codon 23 of pJF8 with the wild- sequence (TCG), allowing the identification of colonies harbo mutagenized plasmids by their Mal$^+$ phenotype on maltose tetr. lium indicator agar supplemented with ampicillin. Plasmids i Mal$^+$ colonies were reintroduced into cells of BAR1091 by ph M13-mediated transduction, and these strains were retained for gle-stranded plasmid DNA preparation and *in vivo* analysis of M. export and processing. DNA sequence analysis performed as descri (24) was used to identify the 1- or 2-base substitutions present at mutagenized codons.

A 20-base mutagenic oligonucleotide coding for the Asp^{-3} alterat (designated *malE22-11*) was utilized to introduce this mutation i plasmids harboring various mutations at the codon for position −1 the MBP signal peptide. Single-stranded DNA of each plasmid s stituted previously at codon −1 was mutagenized as described abo except that mutagenized plasmids were identified solely by D. sequence analysis. Beginning with pJF2 harboring the *malE2* mutation, the oligonucleotide designated PS.11 (Fig. 1) was utiliz to introduce various substitutions into the codon of the *malE* ge encoding the +2 residue. The mutagenesis reactions were perform as described above. Specific mutagenic oligonucleotides (not show also were synthesized to convert the Ala^{-1} codon of plasmid pJF13 an aspartic acid and to delete codons −3 through −1 from plasm pJF2.

Radiolabeling of MBP, Immune Precipitation, SDS-PAGE, an Autoradiography—Cultures were grown to mid-log phase in glyce minimal medium supplemented with ampicillin and induced for sy thesis of MBP by the addition of IPTG to the culture medium. For five min later, cells were labeled with [^{35}S]methionine for 15 min, an the MBP was immune precipitated from solubilized cell extrac using procedures described previously (21). Immune precipitates wer resolved by SDS-PAGE and autoradiography as described previous (21). Pulse-chase experiments (25) and quantitation of the ratio o preMBP to mMBP (26) were also performed as described previousl

Purification and Amino-terminal Analysis of mMBP Species—Cultures of BAR1091 cells harboring the appropriate plasmid wer grown as described above, except that synthesis of MBP was induce in mid-log phase. When the cultures reached early stationary phas the cells were pelleted and washed twice with 10 mM Tris (pH 8.0 Cold osmotic shock as described by Neu and Heppel (27) was used t isolate periplasmic proteins, and crude mMBP was purified on column of cross-linked amylose in 10 mM Tris (pH 7.2) as describe previously (28). The fraction eluted from the column with 10 m maltose, typically in a volume of 7.5 ml, was lyophilized and redi solved in 1 ml of 5% acetic acid with 1.4 guanidinium hydrochlorid The sample was clarified by centrifugation and the entire supernatan injected onto a HPLC (Waters Associates, Milford, MA) equilibrate in 0.05% trifluoroacetic acid. The separation medium was a 300 pore size, 4.6 × 150-mm C-18 reverse phase column (W-Porex, Phenomenex, Rancho Palos Verdes, CA). MBP was eluted from th column with an increasing gradient of acetonitrile at a flow rate o 0.8 ml/min. The eluent was monitored simultaneously at 214 and 25 nm. After an initial fall-through containing various salts, severa minor peaks were observed. MBP was recovered at approximately

```
          -7  -6  -5  -4  -3  -2  -1  +1  +2  +3  +4  +5
         -Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-Glu-Glu-Gly
          TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT

PS.2       TCC GCC TCG WMT CTC GCC AAA ATC
PS.7           GCC TCG GCT CTC WMC AAA ATC GAA GAA GG
PS.11                  T CTC GCC AAA WMC GAA GAA GGT
```

FIG. 1. Oligonucleotides utilized for semirandom mutagenesis of specific codons within the *malE* gene. The portion of the *malE* nucleotide sequence encoding residues −7 to +5 of preMBP is shown. The mutagenic oligonucleotides designated *PS.2*, *PS.7*, and *PS.11* were used to introduce 1- or 2-base substitutions at the codons (*boldfaced*) corresponding to positions −3, −1, or +2. The *letter N* denotes that an equal mixture of all four nucleotides was employed for this step in synthesis of each mutagenic oligonucleotide. The *underlined codon* represents the amber mutation (TAG) in plasmid pJF8.

[1] The abbreviations used are MBP, maltose-binding protein (the prefix "pre" indicates the precursor form of the protein, whereas "m" indicates the mature form); IPTG, isopropyl-1-thio-β-D-galactoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis, HPLC, high performance liquid chromatograph.

40% acetonitrile. The material was lyophilized, resolubilized in 5% acetic acid, and applied to a Polybrene-treated glass fiber filter for 7–15 cycles of amino acid sequence analysis in an Applied Biosystems 470A gas phase sequenator (Foster City, CA) with an on-line Waters HPLC to identify directly phenylthiohydantoin derivatives.

RESULTS

Effect of Single Amino Acid Substitutions at −3 of the MBP Signal Peptide—Oligonucleotide-directed mutagenesis was used to construct a set of derivatives of plasmid pJF2 which differed only in the codon corresponding to position −3 of the MBP signal peptide (see "Materials and Methods"). As a result, a set of plasmids was obtained encoding mutant MBP species in which 1 of 14 different amino acids had been substituted for the alanine normally found at −3 (Table I). Cells harboring these plasmids, as well as all the other derivatives of pJF2 described in this study, exhibited a strongly Mal$^+$ phenotype, indicating that MBP translocation across the cytoplasmic membrane was not strongly affected (6). The effect of each alteration at position −3 on signal peptide processing is shown in Table I. Cells were grown to mid-log phase, induced for synthesis of plasmid-encoded MBP by addition of IPTG, and radiolabeled for 15 min with [^{35}S] methionine. MBP was immune precipitated from solubilized cell extracts and analyzed by SDS-PAGE and autoradiography. Signal peptide processing, as quantitated by measuring directly the radioactivity present in the precursor and mature MBP species, is presented as the percent of the total radiolabeled MBP precipitated which is mMBP. Essentially all the wild-type MBP (98%) was processed to mMBP under these conditions. In addition, MBP harboring glycine, serine, cysteine, threonine, valine, isoleucine, leucine, or proline at −3 was also processed efficiently. Substitution of asparagine for alanine at −3 reduced processing to 25%. The presence of aspartic acid, arginine, histidine, tyrosine, or phenylalanine at −3 resulted in extremely inefficient processing of the signal peptide over the time course of these experiments.

Using a pulse-chase analysis, the kinetics of signal peptide processing was determined for four of the substituted MBP species (Fig. 2). At the earliest chase point (2 min), maturation was complete for both wild-type and Val^{-3} preMBP. The majority of Pro^{-3} preMBP was processed at the 2-min chase point, with the remaining preMBP converted to mMBP by 60 min of chase. Substitution of asparagine at −3 resulted in slow processing of preMBP which was not completed by the last chase point. In contrast to the other MBP species examined, maturation of Arg^{-3} preMBP could not be detected even after a full 60-min chase period.

TABLE I
In vivo processing of preMBP species altered at −3

Substitution	Processed
	%
Ala[a]	98
Gly	98
Ser	99
Cys	99
Thr	98
Val	98
Ile	98
Leu	97
Pro	94
Asn	25
Asp	10
Arg	7
His	7
Phe	7
Tyr	6

[a] Wild-type preMBP.

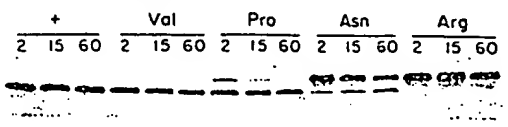

FIG. 2. Kinetics of signal peptide processing in cells synthesizing various preMBP species specifically altered at −3. Glycerol-grown IPTG-induced cells were pulse labeled for 15 s with [^{35}S] methionine, and incubation was continued in the presence of excess cold methionine for the number of min indicated above each lane. The chase was terminated by the addition of an equal volume of ice-cold 10% trichloroacetic acid. The resultant acid precipitates were solubilized, and the MBP was immune precipitated and analyzed by SDS-PAGE and autoradiography. The amino acid indicated above each set of lanes identifies the substitution at −3 for each preMBP species analyzed; + denotes synthesis of wild-type preMBP.

TABLE II
In vivo processing of preMBP species altered at −1

Substitution	Processed	Normal site	Alternate site
	%		
Ala[a]	98	+	
Gly	97	+	
Ser	98	+	
Cys	98	+	
Thr	90	+	+
Asp	91		+
Val	71		+
Asn	41		+
His	35		+
Ile	34		+
Arg	32		+
Leu	31		+
Tyr	19		+
Phe	14		+

[a] Wild-type preMBP.

FIG. 3. Kinetics of signal peptide processing in cells synthesizing various preMBP species specifically altered at −1. The experimental conditions are the same as those described in the legend to Fig. 2. The amino acid indicated above each set of lanes identifies the substitution at −1 for each preMBP species analyzed. + denotes synthesis of wild-type preMBP.

Substitutions at −1 Have Varied Effects on Signal Peptide Processing—Thirteen different amino acid substitutions at the −1 position of the MBP signal peptide were obtained and similarly analyzed (Table II). Precursor MBP species harboring glycine, serine, or cysteine at −1 were virtually completely processed following a 15-min radiolabeling period. In contrast, the other −1 substitutions resulted in efficiencies ranging from 14 to 91%. Kinetic analyses of five representatives from this latter group revealed that processing of each MBP species examined occurred relatively slowly when compared with wild-type preMBP (Fig. 3). The finding that preMBP species with aspartic acid or certain other charged or bulky residues at −1 were processed to significant extents was unexpected. These results suggested that either the statistically based predictions for acceptable residues at the −1 position (10–12) were largely incorrect or that substitutions at −1 were redirecting processing to an alternate site. Within the MBP signal peptide, there is a second potential signal peptidase cleavage site represented by the Ala-*X*-Ala sequence at residues −5 to −3 (see Fig. 1). If this alternate cleavage site were being utilized due to certain substitutions at −1, the resulting

mMBP would be expected to retain an additional 2 amino acids at its amino terminus. The possibility of cleavage at this alternate site was supported by the finding that processing of Arg^{-1}, His^{-1}, and Asn^{-1} preMBP resulted in mMBP species that clearly migrated more slowly than wild-type mMBP when analyzed on a lower percentage polyacrylamide gel (Fig. 4). Mature MBP from the Asp^{-1} and Tyr^{-1} mutants also migrated slower than wild-type mMBP (data not shown), whereas the small amount of mMBP obtained from the Phe^{-1} mutent migrated the same as wild-type mMBP (Fig. 4).

Amino-terminal Analysis of Various mMBP Species—In order to identify conclusively the processing site of four representative MBP species harboring amino acid substitutions at −1, the processed forms of these proteins were purified, and Edman protein degradation (see "Materials and Methods") was utilized to determine the actual amino acid sequence at the amino terminus (data not shown). For cells synthesizing Asp^{-1}, Val^{-1}, and Arg^{-1} preMBP, this analysis clearly indicated that the amino-terminal residue of mMBP was the Leu^{-2}, confirming that cleavage occurred immediately after the Ala-*X*-Ala sequence at residues −5 to −3. When threonine was substituted at −1, two mMBP species were detected. In this instance, approximately 75% of the preMBP processed was cleaved after residue −3, with the remainder being cleaved after the threonine at −1. Recoveries of phenylthiohydantoin derivatives were quantitated at three positions (Leu^1-Lys^1; Lys^3-Glu^3; Gly^1-Leu^1) and were consistent with approximately 3:1 ratios. Note that this was the only preMBP species obtained in this study in which processing at both the normal and alternate cleavage sites could clearly be demonstrated (Table II). For cells synthesizing wild-type Val^{-3} or Pro^{-3} preMBP, only authentically processed mMBP was detected.

Processing of Mutant preMBP Species Lacking the Alternate Cleavage Site—To determine whether −1 substitutions specifically blocked processing at the normal cleavage site without the complication of processing being redirected to an alternate upstream site, the Ala^{-5} of each of these preMBP species was converted to aspartic acid (see "Materials and Methods"). This additional substitution was expected to block cleavage at the alternate site. This substitution did have a minor effect on export and processing of preMBP with alanine (wild-type residue) at −1 (Fig. 5) Aspartic acid at −5 was found to have a similar minor effect on processing of preMBP species having glycine, cysteine, or serine at −1. In contrast, disruption of the alternate site resulted in a total block in processing of preMBP species having aspartic acid, arginine, tyrosine, leucine, or isoleucine at −1, further confirming that the processing observed previously for the corresponding preMBP species having alanine at −5 was effected at the alternate site. Consistent with the amino-terminal analysis of mMBP obtained from the Thr^{-1} mutant, a small amount of processing of the corresponding preMBP species harboring

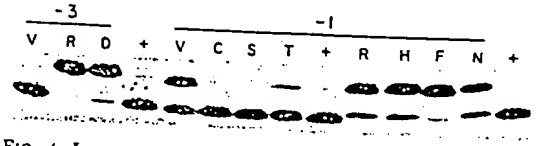

FIG. 4. Immune precipitation of radiolabeled MBP from cells synthesizing various preMBP species specifically altered at either −3 or −1. Glycerol-grown IPTG-induced cells were radiolabeled for 15 min with [^{35}S]methionine. The cells were subsequently solubilized, and the MBP was immunoprecipitated and analyzed by SDS-PAGE and autoradiography as before, except that a 7.5% polyacrylamide gel was employed. The amino acid substitutions at −3 or −1 of preMBP are indicated above each lane by *single letter code*. + denotes synthesis of wild-type preMBP.

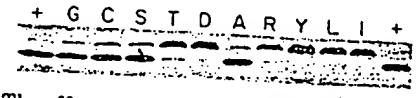

FIG. 5. The effect of amino acid substitutions on process of preMBP lacking the alternate cleavage site. The experim tal conditions are the same as those described in the legend to Fig The amino acid substitutions at −1 of preMBP are indicated ab each lane by *single letter code*. Alanine is the residue found at −1 wild-type preMBP. + denotes synthesis of wild-type preMBP. No that all of the preMBP species except wild-type have an aspartic ac substituted for alanine at −5, which blocks processing at the alterna site.

aspartic acid at −5 was discerned. Although the amount processing at the normal site of this mutant was somewh less than expected, this is most likely due to the alteration the −5 residue which, as noted above, did have some sma effect on processing of otherwise wild-type preMBP. Finall with regard to Phe^{-5} preMBP, repeated attempts to construc a double-mutant MBP species with an aspartic acid at − were unsuccessful.

Substitutions at Position +2 of the Mature Moiety—The efficiency of processing at the alternate site was clearly influenced by the residue occupying the −1 position relative to the normal cleavage site (see Table II and Fig. 3). The substitutions at −1 are actually positioned +2 to the alternate cleavage site. The possibility that substitutions at +2 relative to the normal cleavage site could affect the efficiency of processing at this site was investigated. Once again, *in vitro* mutagenesis was employed to obtain cells synthesizing preMBP species with the following amino acids substituted for the isoleucine normally present at the second position of the mature moiety: arginine, asparagine, aspartic acid, cysteine, histidine, leucine, proline, tyrosine, and valine. In each instance, the signal peptide also had aspartic acid substituted for alanine at −5, thus blocking the alternate cleavage site. The substitutions generated at +2 included residues that had widely varying effects on alternate site processing when they were present at −1 (*e.g.* aspartic acid and tyrosine). Processing of these mutant preMBP species was analyzed by pulse-chase experiments similar to those shown in Figs. 3 and 4. Even at the earliest chase point (in this case 30 s), there were no detectable differences in the processing kinetics observed for any of these mutant preMBP species compared with that discerned for the parental preMBP species with a wild-type mature moiety (data not shown).

Additional Alterations Near the MBP Cleavage Site—It was of interest to determine if the small amount of processing of Asp^{-3} preMBP which could be discerned (see Fig. 4, *third lane*, and Ref. 6) was due to specific recognition of the altered cleavage site by signal peptidase I or by some less specific protease activity in the periplasm. It was found that mMBP could not be detected if preMBP was synthesized with aspartic acid residues at both −3 and −1, indicating that the previously observed maturation of Asp^{-3} preMBP was most likely achieved by specific processing at the normal site. Likewise, maturation of a preMBP species deleted for residues −3 to −1 could not be detected (data not shown).

DISCUSSION

Among prokaryotic signal peptides, only alanine, glycine, serine, leucine, threonine, and valine have been found at position −3 (12). Consistent with the predictions of Perlman and Halvorson (10) and von Heijne (11, 12) this study demonstrated that the presence of any of these residues at −3 was compatible with very efficient preMBP processing. In addition, cysteine at −3 had no noticeable effect on the efficiency

of preMBP processing (see below). Cleavage of Pro^{-3} preMBP also was accomplished with high efficiency, although the kinetics indicated that this precursor species was somewhat less than an optimum substrate for the processing enzyme. Proline is conspicuously absent from the −3 to +1 regions of prokaryotic signal peptides (12). It is frequently encountered at positions −4 to −6, where it is believed to induce a β-turn that may be crucial for efficient presentation of the cleavage site to the processing enzyme (see below). Amino-terminal analysis of the processed forms of Pro^{-3}, Val^{-3}, and wild-type MBP indicated that each species was cleaved exclusively at the normal site.

Kuhn and Wickner (14) reported previously that phage M13 procoat with a phenylalanine at −3 was a very poor substrate for signal peptidase I, and Fikes and Bassford (6) found that preMBP with an aspartic acid substituted for alanine at −3 was translocated efficiently but not processed. In agreement with these results and the von Heijne model (29), the substitution of bulky or charged residues (asparagine, aspartic acid, arginine, histidine, tyrosine, and phenylalanine) at −3 of preMBP resulted in extremely inefficient processing. The small amount of processing observed for Asn^{-3} and Asp^{-3} preMBP was probably due to specific recognition of the altered cleavage site by signal peptidase I since the introduction of an aspartic acid residue at −1 of the Asp^{-3} preMBP or deletion of residues −3 to −1 from otherwise wild-type preMBP resulted in a total block in processing. The signal peptide of unprocessed preMBP is normally highly susceptible to proteolytic degradation, resulting in a polypeptide that migrates identically with mMBP on SDS-PAGE (30, 31). However, it was shown previously that Asp^{-3} preMBP is efficiently translocated into the periplasm but remains firmly anchored to the cytoplasmic membrane by the signal peptide (6). The signal peptides of Asp^{-3} preMBP and the other mutant preMBP species described in this study presumably are shielded from proteolytic attack by their membrane association.

The analysis of changes at the −1 position was somewhat more complicated. Alanine is clearly the residue most frequently encountered at −1, although serine, glycine, and, in one instance, threonine also have been reported (12). Indeed, altered preMBP species having glycine, serine, and also cysteine (see below) substituted for alanine at −1 seemed to be processed as well as wild-type preMBP. Processing of Thr^{-1} preMBP appeared to be nearly as efficient. However, processing at higher than expected efficiencies also was observed for signal peptides having other amino acids substituted at −1. For example, maturation of both Asp^{-1} and Val^{-1} preMBP approached nearly 100% in a pulse-chase experiment. For these and other preMBP species with bulky or charged residues at −1, it was found that processing was not occurring at the normal site. Instead, these preMBP species were primarily cleaved between residues −3 and −2, immediately downstream from the sequence Ala-Ser-Ala that served as an alternate processing site when maturation at the normal site was blocked. Examples of alternate cleavage sites for signal peptidase I in mutant lipoproteins have been described previously (32, 33).

Processing at the alternate site of preMBP was initially indicated by the finding that in some cases, the mature species migrated slightly slower on SDS-PAGE than did the wild-type mMBP. This altered electrophoretic mobility was detected when aspartic acid, arginine, asparagine, histidine, or tyrosine was substituted at position −1 and presumably resulted from the retention of the charged or polar residue at the amino terminus of the mature protein. Amino-terminal analysis subsequently confirmed that the Asp^{-1}, Arg^{-1}, as well as Val^{-1} preMBP species were processed after residue −3. This analysis also revealed that Thr^{-1} preMBP exhibited processing at both the normal and alternate sites, primarily the latter (approximately 75%). Although threonine has been reported at the −1 position of one naturally occurring prokaryotic signal peptide (12), the results obtained with preMBP were more consistent with those of Kuhn and Wickner (14), who found that Thr^{-1} of M13 procoat was a poor substrate for signal peptidase I, both *in vivo* and *in vitro*.

In lieu of determining the cleavage site of each mutant preMBP species altered at −1 by amino-terminal analysis of the purified mature protein, the alternate processing site of these proteins was disrupted by substituting aspartic acid for alanine at −5. In these instances, only preMBP species with alanine, serine, glycine, or cysteine were processed efficiently. A small amount of processing of Thr^{-1} preMBP was discerned; otherwise, disruption of the alternate site appeared to block maturation when aspartic acid, arginine, valine, tyrosine, leucine, isoleucine, histidine, or asparagine was present at −1. These results are very consistent with the conclusions of von Heijne (11) that aromatic, charged and large polar residues are excluded from this position of the cleavage site. As noted previously, attempts to substitute aspartic acid for alanine at −5 of Phe^{-1} preMBP were unsuccessful for an unknown reason. However, it seems highly likely that the small amount of processing observed for this MBP species was also accomplished at the alternate site.

The finding that cysteine residues at either −3 or −1 permitted efficient processing of preMBP was not necessarily surprising. Although not yet encountered in the −4 to +2 region of prokaryotic precursor proteins processed by signal peptidase I, cysteine is a small, neutral residue that is present in this position in many eukaryotic signals (12). Its exclusion from the processing site of prokaryotic signals may be to help distinguish precursor nonlipoproteins from precursor lipoproteins that require a cysteine residue in the +1 position (34). The possibility that mutant preMBP species with cysteine at either −3 or −1 were modified and processed as lipoproteins by signal peptidase II was not specifically investigated. However, the consensus sequence for lipoproteins at residues −3 to +1 (Leu-Ala-Gly-Cys) (34) is considerably different from the corresponding sequences of these proteins (Ser-Ala-Ser-Cys and Ser-Ala-Leu-Cys, respectively; see Fig. 1). Since the mature MBP produced by processing of Cys^{-3} and Cys^{-1} preMBP migrates on SDS-PAGE identically to wild-type mMBP (see Fig. 4), this possibility seems highly unlikely.

Overall, the results obtained experimentally with altered preMBP species agree fairly well with the statistical analyses of prokaryotic signal peptides by von Heijne (11, 12), with the exceptions that cysteine was found to permit efficient processing when present at either −3 and −1, and threonine at −1 resulted in inefficient processing. The data presented here do not address directly the accuracy of the weighted matrix approach for predicting the cleavage sites of prokaryotic signal peptides processed by signal peptidase I (12). Nonetheless, the statistically derived assumptions concerning positions −3 and −1 which constitute a key element to this approach now have been tested directly and found to be correct with only several minor exceptions.

By studying the processing of several prokaryotic proteins in microsomal extracts, Ghrayeb *et al.* (32) concluded that signal peptidase I and eukaryotic signal peptidase possess similar substrate specificities. More recently, Folz *et al.* (35) used site saturation mutagenesis to obtain 13 unique amino acid substitutions at the −1 position of a mutant human

preproapolipoprotein(a-II). Efficient processing in an *in vitro* system was observed with alanine or cysteine in the −1 position. Processing, albeit less efficient, was also discerned with glycine, serine, threonine, or proline at −1. These results may indicate that there are minor differences in the substrate specificities of the prokaryotic and eukaryotic enzymes, although this analysis is somewhat difficult to interpret since kinetic studies were not employed, and an alternative processing site also could be utilized, in this case representing the normal processing site of the wild-type preproapolipoprotein.

In this study, processing at the alternate preMBP cleavage site was only detected when processing at the normal site was blocked or rendered highly inefficient by substitutions at −1. Why is this alternate site not utilized at some detectable frequency in the wild-type signal peptide? In those instances in which the alternate site was cleaved, the processing kinetics were much slower than those exhibited by MBP species processed at the normal site.[2] Thus, in the wild-type signal peptide, extremely rapid cleavage at the normal site may effectively preclude the slower, alternate processing event. Less efficient recognition of the alternate site by signal peptidase I probably results from its proximity to the β-turn structure that represents the boundary between the hydrophobic core and the signal peptidase recognition sequence (10) and that is known, from mutational analyses, to be essential for signal peptide processing (13–15, 36). In prokaryotic signal peptides, the cleavage site is usually separated from the end of the core by six residues. As proposed by Perlman and Halvorson (10), proper juxtaposition of the cleavage site with signal peptidase probably depends strongly on its location relative to the start of the turn. This was demonstrated recently in a convincing fashion for eukaryotic signal peptidase (37). Thus, for preMBP, the alternate processing site seems to be positioned 2 residues too close to the β-turn initiated by the serine at −6 (10) for efficient recognition and cleavage by signal peptidase I.

It was somewhat puzzling to find that there was a wide variation in the efficiency of alternate processing in different mutant preMBP species in which processing at the normal site was blocked. These alterations at −1 relative to the normal cleavage site are actually positioned +2 to the alternate site. Previous studies with M13 procoat indicated that changes at +2 could profoundly affect processing efficiency (38, 39), although stepwise deletions from the cleavage site into the mature region of β-lactamase provided no indication that sequences on the carboxyl side of the cleavage site could influence the reaction kinetics (15). Nine different substitutions for the isoleucine normally present at +2 of preMBP were found to have no noticeable effect on processing at the normal site. This included several amino acids (*e.g.* arginine and tyrosine) that, when present at −1, significantly reduced the efficiency of processing at the alternate site. These results indicated that there probably is not a strong restriction *per se* on the amino acid that can occupy the +2 position relative to the cleavage site. A recent study by Duffaud and Inouye (40) demonstrated that mutational alterations in the adjacent mature region which decreased the probability of a turn structure at the cleavage site itself could strongly affect the processing efficiency of an OmpA-staphylococcal nuclease chimeric protein expressed in *E. coli*. The possibility was considered that the alternate preMBP cleavage site, co with the normal site, was much more sensitive to cha +2 which affected the turn probability through that However, there does not appear to be a strong cor between alternate site processing efficiencies and the sity of the residue substituted at −1 of the normal c site to participate in a turn structure.[3] This is being gated further.

Finally, what is the significance of the finding of a nate processing site in the preMBP? Its presence may sent the rule rather than the exception. The LamB, gala binding protein, and ribose-binding protein signal pe also harbor the sequence Ala-*X*-Ala-*X*-Ala at positions −1 (41). Overall, the frequency of compatible residues and −3 for constituting potential alternate processing s prokaryotic signal peptides is quite high (41). There n circumstances in which the presence of alternate cl sites ensures efficient processing. It may also ensure pr ing in the event of a mutational alteration elsewhere signal which shifts the proximity of the core to the n cleavage site. The existence of alternate cleavage sites appears to reflect a general redundancy in the features have been identified as being important to promoting pr export. For example, many prokaryotic signal peptides more than 1 basic residue in the amino-terminal hydro segment, whereas several studies have shown that a s such residue is sufficient (42, 43). Likewise, the hydroph cores of most signal peptides appear to be longer than req (44, 45). Although this built-in redundancy may seem s fluous in the laboratory setting, nature may have deem important in maintaining protein translocation and s peptide cleavage as rapid and highly efficient cellular p esses.

Acknowledgments—We thank Paul Ray, Inderjit Dev, and Weiss for stimulating discussions and for critically reading the uscript.

REFERENCES

1. Duffaud, G. D., Lehnhardt, S. K., March, P. E., and Inouye (1985) *Curr. Top. Membr. Transp.* 24, 65–104
2. Verner, K., and Schatz, G. (1988) *Science* 241, 1307–1313
3. von Heijne, G. (1985) *J. Mol. Biol.* 184, 99–105
4. Inouye, M., and Halegoua, S. (1980) *Crit. Rev. Biochem.* 7, 371
5. Dalbey, R. E., and Wickner, W. (1985) *J. Biol. Chem.* 15925–15931
6. Fikes, J. D., and Bassford, P. J., Jr. (1986) *J. Bacteriol.* 1 2352–2359
7. Evans, E. A., Gilmore, R., and Blobel, G. (1986) *Proc. Natl. A Sci. U. S. A.* 83, 581–585
8. Baker, R. K., and Lively, M. O. (1987) *Biochemistry* 26, 8 8567
9. Ray, P., Dev. I, MacGregor, C., and Bassford, P. J., Jr. (1 *Curr. Top. Microbiol. Immunol.* 125, 75–102
10. Perlman, D., and Halvorson, H. O. (1983) *J. Mol. Biol.* 167, 3 409
11. von Heijne, G. (1983) *Eur. J. Biochem.* 116, 17–21
12. von Heijne, G. (1986) *Nucleic Acids Res.* 14, 4683–4690
13. Koshland, D., Sauer, R. T., and Botstein, D. (1982) *Cell* 30, 9 914
14. Kuhn, A., and Wickner, W. (1985) *J. Biol. Chem.* 260, 159 15918
15. Plückthun, A., and Knowles, J. R. (1987) *J. Biol. Chem.* 2 3951–3957
16. Bedouelle, H., Bassford, P. J., Jr., Fowler, A. V., Zabin, Beckwith, J., and Hofnung, M. (1980) *Nature* 285, 78–81
17. Dean, D. A., Fikes, J. D., Gehring, K., Bassford, P. J., Jr., a Nikaido, H. (1989) *J. Bacteriol.* 171, 503–510

[2] Although the relatively slow rate of processing observed for cleavage at the alternate site actually could have resulted from a delay in translocation across the cytoplasmic membrane, recent experiments indicated that the rate of translocation was not adversely affected by mutational alterations at either the −3 or −1 position (G. Barkocy-Gallagher and P. Bassford, unpublished data).

[3] G. Barkocy-Gallagher and P. Bassford, unpublished data.

18. Rasmussen, B. A., MacGregor, C. A., Ray, P. H., and Bassford, P. J., Jr. (1985) *J. Bacteriol.* 164, 665–673
19. Vieira, J., and Messing, J. (1987) *Methods Enzymol.* 153, 3–11
20. Miller, J. H. (1972) *Experiments in Molecular Genetics*, pp. 431–435, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Emr, S. D., and Bassford, P. J., Jr. (1982) *J. Biol. Chem.* 257, 5852–5860
22. Zoller, M. J., and Smith, M. (1985) *DNA (N. Y.)* 3, 479–488
23. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* 154, 367–382
24. Hutchison, C. A., III, Nordeen, S. K., Vogt, K., and Edgell, M. H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 710–714
25. Ryan, J. P., and Bassford, P. J., Jr. (1985) *J. Biol. Chem.* 260, 14832–14837
26. Collier, D. N., Bankaitis, V. A., Weiss, J. B., and Bassford, P. J., Jr. (1988) *Cell* 53, 273–283
27. Neu, H. C., and Heppel, L. A. (1965) *J. Biol. Chem.* 240, 3685–3692
28. Ferenci, T., and Klotz, U. (1978) *FEBS Lett.* 94, 213–217
29. von Heijne, G. (1985) *Curr. Top. Membr. Transp.* 24, 151–179
30. Randall, L. L. (1983) *Cell* 33, 231–240
31. Dierstein, R., and Wickner, W. (1985) *J. Biol. Chem.* 260, 15919–15924
32. Ghrayeb, J., Lunn, C. A., Inouye, S., and Inouye, M. (1985) *J. Biol. Chem.* 260, 10961–10965
33. Hayashi, S., Chang, S.-Y., Chang, S., and Wu, H. C. (1986) *J. Bacteriol.* 165, 678–681
34. Wu, H. C., and Tokunaga, M. (1986) *Curr. Top. Microbiol. Immunol.* 125, 128–157
35. Folz, R. J., Nothwehr, S. F., and Gordon, J. I. (1988) *J. Biol. Chem.* 263, 2070–2078
36. Kadonaga, J. T., Plückthun, A., and Knowles, J. R. (1985) *J. Biol. Chem.* 260, 16192–16199
37. Nothwehr, S. F., and Gordon, J. I. (1989) *J. Biol. Chem.* 264, 3979–3987
38. Boeke, J. D., Russel, M., and Model, P. (1980) *J. Mol. Biol.* 144, 103–116
39. Russel, M., and Model, P. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 1717–1721
40. Duffaud, G., and Inouye, M. (1988) *J. Biol. Chem.* 263, 10224–10228
41. Sjöström, M., Wold, S., Wieslander, A., and Rilfors, L. (1987) *EMBO J.* 6, 823–831
42. Inouye, S., Soberon, X., Franceschini, T., Nakamura, K., and Inouye, M. (1982) *Proc. Natl. Acad. Sci. U. S. A.* 79, 3138–3141
43. Puziss, J. W., Fikes, J. D., and Bassford, P. J., Jr. (1989) *J. Bacteriol.* 171, 2303–2311
44. Ferenci, T., and Silhavy, T. J. (1987) *J. Bacteriol.* 169, 5339–5342
45. Fikes, J. D., Bankaitis, V. A., Ryan, J. P., and Bassford, P. J., Jr. (1987) *J. Bacteriol.* 169, 2345–2351

THIS PAGE BLANK (USPTO)